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Heterologous mRNA-protein vaccination with Tc24 induces a robust cellular immune response against *Trypanosoma cruzi*, characterized by an increased level of polyfunctional CD8$^+$ T-cells

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A R T I C L E I N F O

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A B S T R A C T

Tc24 is a *Trypanosoma cruzi*-derived flagellar protein that, when formulated with a TLR-4 agonist adjuvant, induces a balanced immune response in mice, elevating IgG2a antibody titers and IFN-γ levels. Furthermore, vaccination with the recombinant Tc24 protein can reduce parasite levels and improve survival during acute infection. Although some mRNA vaccines have been proven to elicit a stronger immune response than some protein vaccines, they have not been used against *T. cruzi*. This work evaluates the immunogenicity of a heterologous prime/boost vaccination regimen using protein and mRNA-based Tc24 vaccines. Mice (C57BL/6) were vaccinated twice subcutaneously, three weeks apart, with either the Tc24-C4 protein + glucopyranosyl A (GLA)-squalene emulsion, Tc24 mRNA Lipid Nanoparticles, or with heterologous protein/mRNA or mRNA/protein combinations, respectively. Two weeks after the last vaccination, mice were euthanized, spleens were collected to measure antigen-specific T-cell responses, and sera were collected to evaluate IgG titers and isotypes. Heterologous presentation of the Tc24 antigen generated antigen-specific polyfunctional CD8$^+$ T cells, a balanced Th1/Th2/Th17 cytokine profile, and a balanced humoral response with increased serum IgG, IgG1 and IgG2c antibody responses. We conclude that heterologous vaccination using Tc24 mRNA to prime and Tc24-C4 protein to boost induces a broad and robust antigen-specific immune response that was equivalent or superior to two doses of a homologous protein vaccine, the homologous mRNA vaccine and the heterologous Tc24-C4 Protein/mRNA vaccine.

1. Introduction

Chagas disease (CD) is a parasitic disease caused by the protozoan parasite *Trypanosoma cruzi* (Álvarez-Hernández et al., 2021). The World Health Organization (WHO) estimates that 6 to 7 million people worldwide are infected with *T. cruzi* (World Health Organization, 2015). Although endemic in Latin America, CD can now also be found in other populations worldwide (Álvarez-Hernández et al., 2021; World Health Organization, 2015), including in the United States, where the Centers for Disease Control and Prevention (CDC) estimate that almost 37,000 people live with CD in Texas alone (Hotez, 2018). It is reasonable to consider that part of these cases may be imported from endemic areas,

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although local transmission in the state has been reported since the 1940s with endemic human CD cases associated with local triatomines (Garcia et al., 2015). The Global Burden of Disease Study 2019 estimated that CD is responsible for 275,000 disability-adjusted life years (DALY) due to its substantial morbidity and premature mortality (Lee et al., 2013). Available drugs for CD treatment, such as Nifurtimox and Benznidazole (BZN), are only effective in the early infection phase, in addition to having several side effects (Pérez-Molina and Molina, 2018). Moreover, the drugs often fail to halt the progression of chronic Chagasic cardiomyopathy (CCC) in individuals with persistent parasites (Morillo et al., 2015). Therefore, efforts must be focused on the development of more efficient and less toxic drugs and the development of prophylactic and therapeutic vaccines, especially for CCC (Bivona et al., 2020).

CD is characterized by two clinical phases, acute and chronic. The acute stage lasts 2 to 4 months and is asymptomatic or non-specific in 95% of the cases. Chronically infected individuals either remain in the asymptomatic or indeterminate stage (70–90%) or transition to the determinate stage, where clinical manifestations develop in 10–30% of individuals, characterized by cardiac and/or digestive complications (Álvarez-Hernández et al., 2021). While the immune system attempts to control the infection by generating lytic antibodies, $CD8^+$ T cytotoxic and $CD4^+$ T helper IFN-γ producing lymphocytes (Tarleton, 2007; Lidani et al., 2017), it often cannot fully resolve the infection (Kumar and Tarleton, 1998). In the chronic stage, it has been demonstrated that patients with cardiac symptoms present an immunological profile comprised of an overall Th1 cytokine polarization (IFN-γ, TNF, IL-2, IL-6, IL-9, IL-12) with low levels of Th2 cytokines (IL-4, IL-5, IL-10, IL-13) (Cristovão-Silva et al., 2021; Poveda et al., 2014). The opposite is observed in the indeterminate form, with patients having higher Th2 cytokine levels overall (Chevillard et al., 2018). A Th1 skewed proinflammatory response can lead to tissue damage, with cardiac inflammation and fibrosis (Chaves et al., 2019). Additionally, it has been shown that patients with the milder CD cardiac form have a higher production of IL-10 and IL-17, while the most severe form of the disease is associated with lower levels of IL-17 (Guedes et al., 2012; Rodrigues et al., 2012). Altogether, these studies point to the importance of regulating pro- and anti-inflammatory immune profiles in the context of CD. This includes eliciting a balanced Th1/Th2/Th17 immunity that, while destroying parasites that persist, does not exacerbate host inflammatory or fibrotic responses.

These insights were used to design a first-generation recombinant protein vaccine candidate based on the *T. cruzi*-derived flagellar antigen Tc24, adjuvanted with a TLR4 agonists such as monophosphoryl-lipid A (MPLA) or synthetic equivalents (e.g., glucopyranosyl lipid A (GLA) or E6020), induced protection in mice. It was shown to be an effective vaccine by increasing host survival during acute infection (Martinez-Campos et al., 2015). The immunogenicity of a modified Tc24 protein with increased stability, designated Tc24-C4 (Seid et al., 2017), and adjuvanted with E6020 was tested in naïve non-human primates, displaying specific IgG production and IFNγ-producing $CD4^+$ cells (Dumonteil et al., 2020). Tc24-C4 in a stable emulsion with E6020 adjuvant was shown to be an effective vaccine as a therapeutic intervention during *T. cruzi* acute and chronic infection, reducing cardiac inflammation and fibrosis (Cruz-Chan et al., 2021; Barry et al., 2019). In acutely infected mice, the vaccination allowed a reduced dose of BZN chemotherapy and improved host survival, inducing antigen-specific IL-17A, IL-23, and IL-22 secretion, and increasing $CD8^+$ T and *T. cruzi*-specific IFNγ-producing $CD4^+$ cells (Cruz-Chan et al., 2021). During the chronic phase, the vaccination in *T. cruzi* infected mice increased antigen-specific IFNγ-producing cells and IFN-γ levels, along with IgG2a antibody titers (Barry et al., 2019).

A new generation of antiviral RNA-based vaccines has shown the ability to elicit protective immunity, inducing strong antigen-specific $CD8^+$ T-cell and potent $CD4^+$ T-cell responses (Chahal et al., 2017; Pardi et al., 2018). Furthermore, with relative ease of design and manufacture, RNA vaccines have proven their safety and efficacy during the COVID-19 pandemic (Versteeg et al., 2019; Zhang et al., 2019; Muik et al., 2022). Consequently, there is a rapidly growing number of mRNA vaccine development programs for both viral and non-viral infectious diseases. For example, the *Plasmodium* circumsporozoite protein (PfCSP) when evaluated as an mRNA malaria vaccine showed immunogenic and protective potential (Mallory et al., 2021). Also, a promising mRNA vaccine against Zika virus that encodes the pre-membrane and envelope (prM–E) glycoproteins of the virus has been reported (Medina-Magües et al., 2021). Nevertheless, there are still no studies of mRNA vaccines for CD.

Here we present immunogenicity of a CD mRNA vaccine based on the Tc24 antigen. The vaccine is tested alone and in a heterologous vaccination scheme combined with the Tc24-C4/GLA-SE protein vaccine. While heterologous vaccination may increase development costs and complicate production, it may also offer superior immunity. This may be of specific interest for vaccine development focused on complex pathogens such as parasites (Chahal et al., 2017); heterologous vaccination has been reported to successfully generate protection against *Leishmania donovani* infection when mice were immunized with LEISH-F2-expressing RNA vaccine followed by a subunit vaccine (Duthie et al., 2018). Furthermore, a DNA-prime/chimpanzee adenovirus 63 (ChAd63) boost vaccine encoding *Plasmodium falciparum* CSP, Pf apical membrane antigen-1 (PfAMA1), and a pre-erythrocytic stage (ME-TRAP) antigens exhibited significant protective efficacy against controlled human malaria infection (Sklar et al., 2021). A malaria vaccine against *Plasmodium vivax* was also evaluated. Human serotype 5 (AdHu5) and chimpanzee serotype 68 (AdC68) adenovirus vectors expressing CSP were used to prime mice boosted twice with yPvCSP, two chimeric proteins containing the three central repeat regions of different CSP alleles. This vaccination regimen induced high levels of specific antibody production and could significantly delay the time to reach 1% parasitemia in rodent malaria model challenge studies (De Camargo et al., 2018). In a CD model, DNA prime/protein boost protocols of Traspain, a trivalent immunogen, enhanced protection levels in vaccinated mice against *T. cruzi* infection by stimulating polyfunctional $CD4^+$ and $CD8^+$ T-cell-mediated immunity (Sanchez Alberti et al., 2020).

Considering the complexity of CD and despite the strong results using the heterologous strategy of DNA prime/protein boost, a commercial vaccine remains elusive since no DNA vaccines have yet achieved licensure. Taking these studies into account, this work aimed to evaluate, for the first time, an mRNA-based vaccine for CD in homologous and heterologous prime/boost vaccination regimens using protein and mRNA-based Tc24 vaccines in C57BL/6 mice. Such studies could be built on a Tc24-C4 recombinant antigen that is expected to enter the clinic soon as a monovalent therapeutic CD vaccine (Jones et al., 2022). This study shows how the presentation of the same antigen as either protein or mRNA generates distinct immune profiles in mice, and how a heterologous vaccination scheme can contribute to a balanced humoral and cellular immune response.

2. Material and methods

2.1. Mice

Female C57BL/6 mice (Jackson), aged 5–8 weeks, were used for the studies. Animal experiments were performed in full compliance with the Guide for the Care and Use of Laboratory Animals, 8th edition (National Research Council, 2011), under a protocol approved by Baylor College of Medicine's Institutional Animal Care and Use Committee (IACUC).

2.2. mRNA production

Tc24 sequences were cloned into pTNT (Promega) to facilitate mRNA transcription. Codon usage was optimized to improve ribosome binding and expression efficacy. A KOZAK sequence (GCCRCCAUGG) was added ahead of the Tc24 ORF to assist in the initiation of the

translation process. The plasmid was cloned in *Escherichia coli*. To produce RNA, 200 μg of plasmid encoding Tc24 were first linearized using BamHI. The plasmid was then purified on MaXtract High-Density columns (Qiagen) and by precipitation in ethanol and quantified by absorbance at 260 nm on a Nanodrop spectrophotometer. The linearized plasmid was stored at -20 °C until use. Subsequently, RNA was transcribed using RiboMAX Large Scale RNA Production Systems (Promega), replacing uridine for modified 5-Methoxy-UTP (Trilink). These RNAs were purified by precipitation in ammonium acetate and capped using ScriptCap™ m7G Capping System (CellScript) to enhance cell RNA stability and translation. The mRNA was quantified by absorbance at 260 nm on a Nanodrop spectrophotometer. All RNAs were stored at -80 °C until use. The integrity, quantity, and quality of the transcribed RNA were assessed through denaturing agarose gel electrophoresis. Protein translation was verified by *in vitro* translation in a mouse dendritic cell line (DC 2.4, Millipore Sigma) followed by SDS-gel electrophoresis and western blotting (**Supplement data 1)**.

2.3. Lipid Nanoparticles synthesis

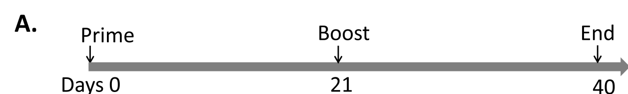
Lipid Nanoparticles (LNPs) were synthesized using the microfluidic benchtop system NanoAssemblr (Precision Nanosystems) DLin-MC3-DMA (the ionizable lipid that allows conjugation with the mRNA), DSPC, Cholesterol, and DMG-PEG2000 (molar ratios 50:10.5:38:1.5, respectively) were dissolved in ethanol for a 10 mM total lipidic concentration. Tc24 mRNA was dissolved in the aqueous phase (50 mM sodium acetate buffer, pH = 4, adjusted by adding 17 M acetic acid dropwise), keeping a ratio of 1:20 between the mass of lipids and that of mRNA. Before synthesis, the organic phase was heated for 3 min at 45 °C, while the aqueous phase was briefly (<60 s) warmed up to 42 °C. The synthesis was carried out with a flow ratio (FR = organic phase:aqueous phase) of 1:3 and a total flow rate (the flow rate at which the formulation is dispensed) of 2 mL/min.

2.4. Vaccination

Female C57BL/6 mice (n = 5) were vaccinated subcutaneously two times, three weeks apart, with the selected vaccine (Days 0 and 21) (Fig. 1A and B). Blood was collected before the boost and at the time of euthanasia. Serum was harvested from whole blood to measure antibody responses. Two weeks after the final vaccination, the mice were humanely euthanized blood and spleens were collected to measure antigen-specific humoral and T-cell responses. Two independent experiments were performed.

We compared four different vaccination strategies: 1) homologous vaccination with Tc24-C4 protein + GLA-Squalene Emulsion (GLA-SE) adjuvant (prime and boost), 2) homologous vaccination with Tc24 mRNA LNPs (prime and boost), 3) heterologous vaccination with the Tc24-C4 protein vaccine (prime), followed by the Tc24 mRNA vaccine (boost), and 4) heterologous vaccination with the Tc24 mRNA vaccine (prime) followed by the Tc24-C4 protein vaccine (boost). Tc24-C4 protein was expressed and purified as previously described and combined with the TLR4 agonist adjuvant GLA in a stable squalene emulsion (AAHI) (Seid et al., 2017). The control groups were 1) Saline, 2) empty LNPs, and 3) GLA-SE alone.

A.



B.

Group	Antigen or Control	Antigen Dose (μg)	Adjuvant Molecule	Adjuvant Dose (μg)	Delivery system
1	Saline	-	-	-	-
2	Empty LNPs	-	-	-	LNPs
3	GLA-SE	-	GLA-SE	5	-
4	Tc24-C4 Protein + GLA-SE	25	GLA-SE	5	-
5	Tc24 mRNA	10	-	-	LNPs
6	Tc24 mRNA Prime/	10	-	-	LNPs
	Tc24-C4 Protein + GLA-SE Boost	25	GLA-SE	5	-
7	Tc24-C4 Protein + GLA-SE Prime/	25	GLA-SE	5	-
	Tc24 mRNA Boost	10	-	-	LNPs

Fig. 1. Groups and Vaccination Scheme. Mice were treated according to the treatment scheme shown in panel **(A)** and were assigned to the treatment groups listed in panel **(B)**.

2.5. Splenocyte preparation and in vitro restimulation

Spleens were rinsed in sterile PBS, transferred to a gentleMACS C Tube containing 3 mL sterile PBS, and homogenized on a gentleMACS Dissociator (Miltenyi Biotech). Red blood cells from the spleen homogenates were lysed with ACK lysis buffer (Lonza). The lysis solution was diluted 5-fold with RPMI medium supplemented with 10% fetal bovine serum FBS, 1 × penicillin-streptomycin (Pen-Strep), and l-glutamine (cRPMI medium). Splenocytes were then pelleted by centrifugation for 5 min at 300×*g*. Next, splenocytes were resuspended in 5 mL cRPMI medium and passed through 40 μm strainers (BD Biosciences). Cells were counted using acridine orange-propidium iodide (AOPI) live/dead dye and a Cellometer Auto 2000 (Nexcelom Bioscience) automated cell counter. Then, for each sample, 1×10^6 live splenocytes were incubated in a 96-well non-tissue culture plate with either 10 μg/mL recombinant Tc24-C4 protein, 20 ng/mL phorbol 12-myristate 13-acetate (PMA)–1 μg/mL ionomycin, or cRPMI medium (unstimulated)only for 48 h at 37 °C in 5% CO_2.

2.6. Flow cytometry analysis

Cells were collected 48 h post restimulation for flow cytometry labeling. To measure CD4-and CD8-specific responses, restimulated splenocytes were collected, washed with PBS, and stained with Live/Dead fixable Near-IR viability dye, anti-CD3e Brilliant Violet 421 (BV-421), anti-CD4 Alexa Fluor 700, and anti-CD8a peridinin chlorophyll protein (PerCP)-Cy5.5. Activation of T-cells was evaluated with the surface anti-CD25 fluorescein isothiocyanate (FITC) (Supplementary Table 1). In addition, 4.1 μg/mL brefeldin A was added to splenocytes for the last 5 h of incubation to evaluate intracellular cytokine production. Splenocytes were stained for the surface markers described above, fixed, and permeabilized with BD Cytofix/Cytoperm, according to the manufacturer's instructions. Permeabilized splenocytes were stained with anti-IFN-γ allophycocyanin (APC) and anti-TNF phycoerythrin (PE), IL-17A Brilliant Violet (BV)-610, and IL-2 BV-510. Samples were acquired on an Attune instrument, and at least 100,000 total events in a live gate were analyzed using Venturi One version 6 software (Applied cytometry). To evaluate antigen-specific responses, the percentage of unstimulated cells served as background and was then subtracted from the percentage of the antigen-stimulated cells of the same mouse.

2.7. Multiplex analysis of secreted cytokines by Luminex

Supernatant of restimulated splenocytes was collected after 48 h and frozen at -80 °C until use. The evaluation of the secreted levels of IL-2, IL-4, IL-6, IL-10, IL-13, IL-17A, IL-22, IFN-γ, and TNF-α from supernatants was conducted using a Luminex-based assay, as previously described (Versteeg et al., 2017). The levels of IL-13 and IL-17A concentrations were below the detection limit. The values of cytokines measured from splenocytes restimulated with only medium served as background. For each sample, cytokine values measured from medium only stimulated cells were subtracted from antigen stimulated cells to obtain antigen-specific cytokine values.

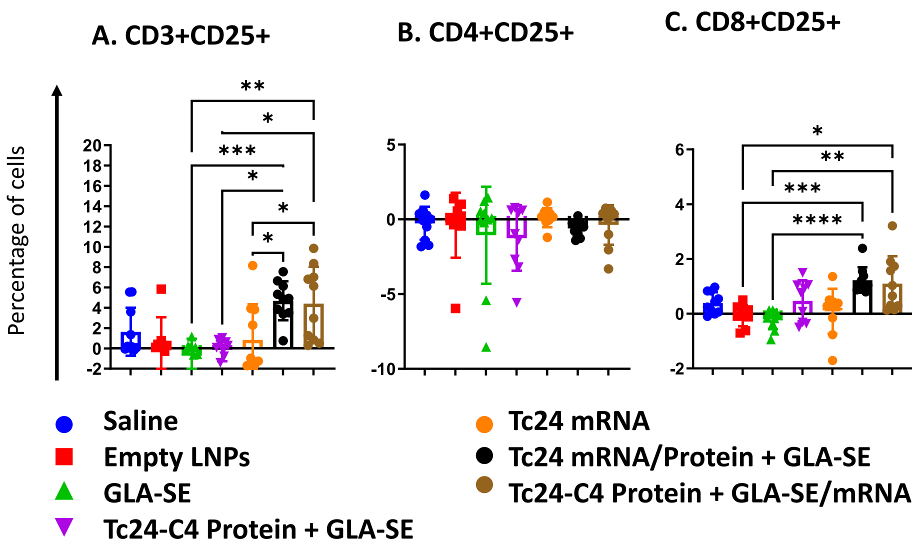


Fig. 2. Expression of the T-cell activation marker CD25 in T-cells. Flow cytometry analysis of splenocytes restimulated with 10 μg/μL Tc24-C4 protein for 48 h. **A.** Analysis of activated $CD3^+CD25^+$ T-cells **B.** activated $CD4^+CD25^+$ T-cells and C. activated $CD8^+CD25^+$ T-cells. The results are from two independent experiments. Each point represents an individual mouse from the two replicate studies (n = 10); horizontal lines denote median values; significance was calculated by the Kruskal-Wallis test with Dunn's correction for multiple comparisons. P-values of ≤0.05, ≤0.01, ≤0.001, and ≤0.0001 are represented as one, two, three, and four symbol characters, respectively.

2.8. ELISA Tc24-specific IgG, IgG1, and IgG2c

Indirect ELISAs were conducted to measure the Tc24-specific antibody titers from serum. 96-well NUNC ELISA plates were coated overnight at 4 °C with 0.3125 μg/mL Tc24-C4 in 1x coating buffer. The coating buffer (10x coating solution, KPL) was discarded the following day, and plates were blocked with assay buffer (0.1% BSA in PBST, 1X PBS in 0.05% of Tween 20) for 2 h at room temperature. Mouse serum was two-fold serially diluted in an assay buffer, starting at 1:200. As a negative control, pooled naïve mouse sera were diluted to 1:200, and 1:1600 diluted Tc24-C4 pooled antisera were used as a positive control. ELISA plates were washed using a BioTek 405 TS plate washer and PBST. Diluted mouse serum and the controls were added to the washed ELISA plates in duplicate at 100 μL/well, followed by an incubation of 2 h at room temperature. Next, plates were washed four times, followed by adding 100 μL/well 1:6000 diluted goat anti-mouse IgG HRP (Lifespan Bioscience), goat anti-mouse IgG1 HRP (Lifespan Bioscience), or goat anti-mouse IgG2c HRP (Lifespan Bioscience). After 1 h of incubation at room temperature, ELISA plates were washed five times, followed by a 15-min incubation of 100 μL/well TMB substrate (KPL). Finally, the reaction was stopped by adding 100 μL/well 1M HCl, and the absorbance at 450 nm was read using an Epoch 2 spectrophotometer (Biotek).

Duplicate values of the measured O.D. at 450 nm were averaged for data analysis. The titer cutoff value was calculated as follows: titer cutoff = average negative control +3 x standard deviation of the negative control. The titer was determined for each mouse serum sample by taking the corresponding dilution factor of the highest dilution with an average O.D. value above the titer cutoff. If a sample did not show an average O.D. value above the titer cutoff at 1:200, an arbitrary titer value of 67 was assigned (baseline).

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 9.3.1 for Windows (GraphPad Software, San Diego, California, USA). Significance was calculated by nonparametric Kruskal-Wallis H test, one-way analysis. All vaccine groups were compared to the saline, GLA-SE, or empty LNP controls. Additionally, heterologous vaccination groups were compared to the homologous vaccination groups and between each other. P values ≤ 0.05 were considered significant. In the figures, P-values of ≤0.05, ≤0.01, ≤0.001, and ≤0.0001 are represented as one, two, three, and four symbol characters, respectively.

3. Results

3.1. Heterologous vaccination improves T-cell immune responses

To characterize the immune response to the homologous or heterologous vaccination schemes, mice were immunized twice with either the Tc24-C4 protein, Tc24 mRNA, or with heterologous protein/mRNA or mRNA/protein combinations. Stimulation of lymphocytes from mice vaccinated with protein and mRNA showed upregulation of various cell surface markers, such as CD69 (early) and CD25 (late). T-cell activation plays a crucial role in coordinating the immune response and killing cells infected by pathogens (Reddy et al., 2004). Since mRNA vaccines have been shown to evoke strong T-cell responses (Vogel et al., 2021; Zhang et al., 2020), we focused on the expression of $CD25^+$ as a marker of T-cell activation (Fig. 2).

Mice vaccinated with heterologous vaccines showed a notable increase in the percentage of activated T-cells from splenocytes ($CD3^+CD25^+$) compared to controls or the homologous Tc24-C4 protein groups (Fig. 2A). In addition, both heterologous vaccines induced higher levels of activated $CD8^+CD25^+$ cells (Fig. 2C). Homologous vaccination schemes with Tc24 mRNA or Tc24-C4 protein vaccines did not induce significantly higher numbers of $CD3^+CD25^+$ cells (Fig. 2A). Finally, $CD4^+CD25^+$ T-cells were not significantly different between groups (Fig. 2B).

Additionally, when we evaluated cytokine-producing $CD4^+$ and $CD8^+$ T-cells (Fig. 3), we observed an increase in the production of IFN-γ in $CD4^+$ T-cells in both heterologous vaccination groups in comparison with the control groups, indicating a stronger antigen-specific immune response when the Tc24 antigen is presented as both protein and mRNA. There were no significant differences in the production of cytokines IL-2 (Fig. 3B), IL-17A (Fig. 3C), and TNF-α (Fig. 3D) in the $CD4^+$ T-cells. Vaccination using the heterologous vaccination strategy as well as using two doses of the Tc24-C4 protein induced significantly increased $CD8^+$IFNg T-cells compared to controls or to two doses of the Tc24 mRNA vaccine (Fig. 3E). Similarly, heterologous vaccination induced significantly increased numbers of $CD8^+$IL-2 cells compared to either homologous vaccine or the controls (Fig. 3F). Finally, there was no

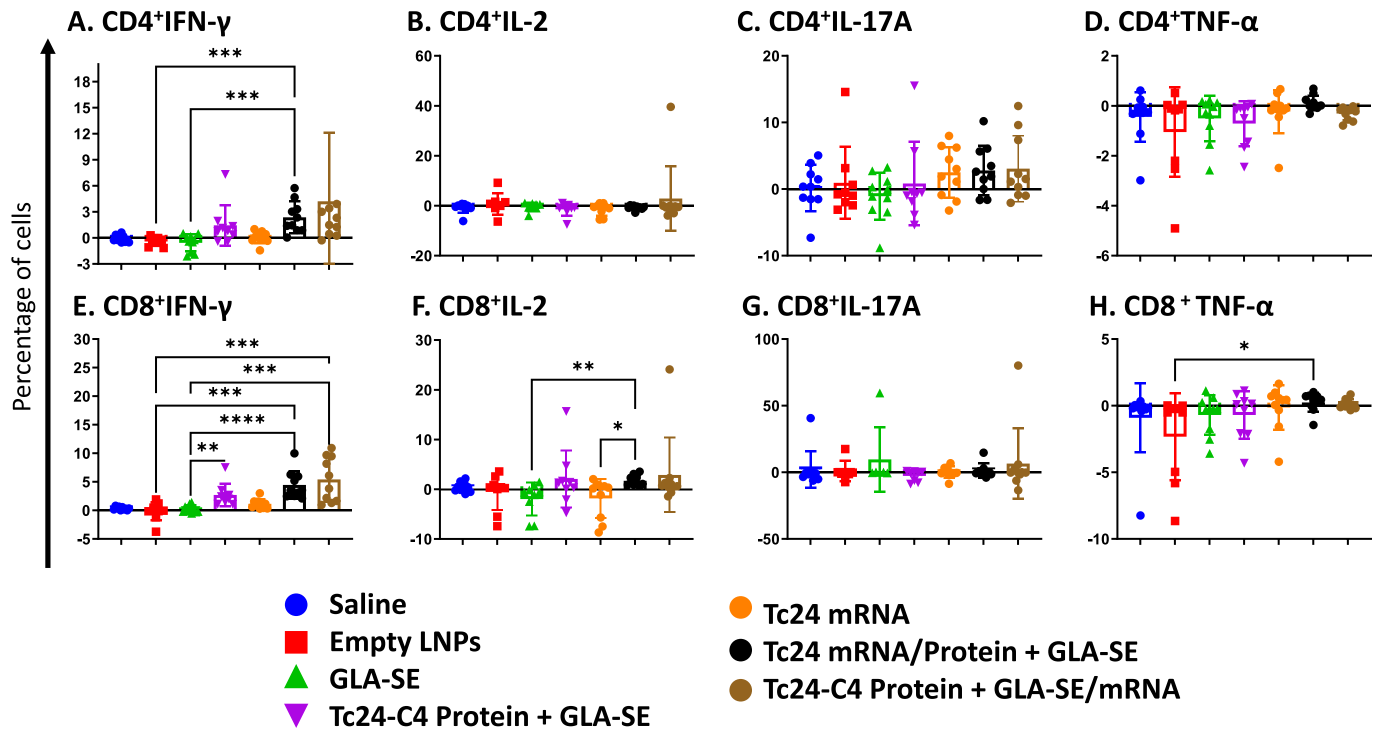


Fig. 3. Cytokine production in CD4$^+$ and CD8$^+$ T-cells after restimulation with Tc24-C4 protein for 48 h. Production of cytokines in CD4$^+$ T-cells. **A.** IFN-γ, **B.** IL-2, **C.** IL-17A **D.**TNF-α, and the CD8$^+$ T-cell **E.** IFN-γ, **F.** IL2 **G.** IL-17A, and **H.** TNF-α. The results are from two independent experiments. Each point represents an individual mouse (n = 10); horizontal lines denote median values; significance was calculated by the Kruskal-Wallis test with Dunn's correction for multiple comparisons. P-values of ≤0.05, ≤0.01, ≤0.001, and ≤0.0001 are represented as one, two, three, and four symbol characters, respectively.

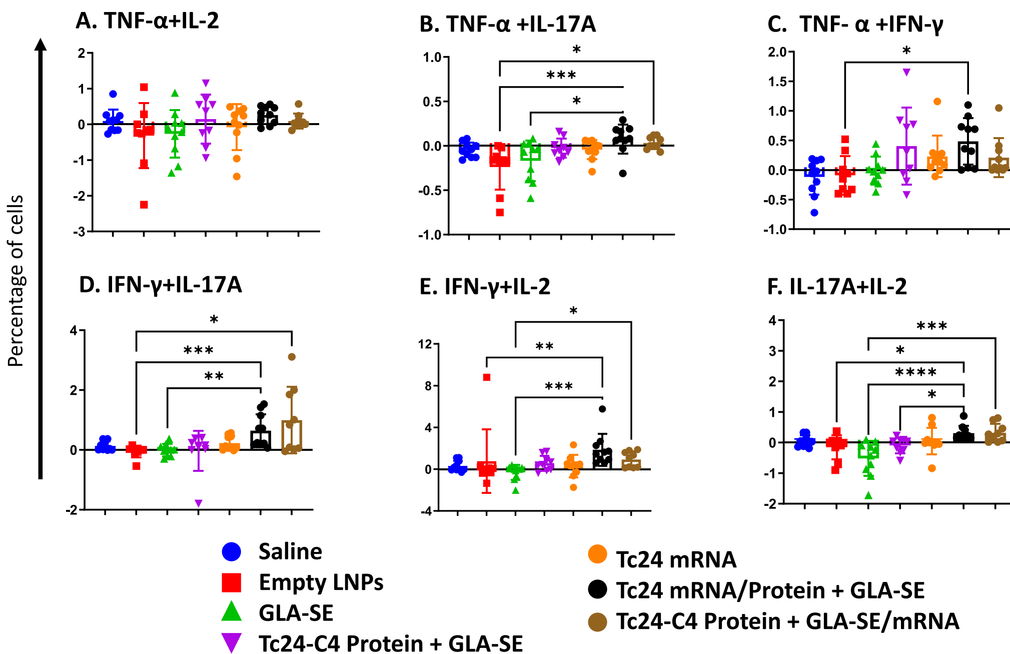


Fig. 4. Polyfunctional CD8$^+$ T-cell responses after vaccination. Flow analysis of cytokine co-production by splenocytes of vaccinated mice. **A.** TNF-α +IL-2, **B.** TNF-α +IL-17A, **C.** TNF-α +IFN-γ **D.** IFN-γ+IL-17A, **E.** IFN-γ+IL2, **F.** IL17A + IL2. The results are from two independent experiments. Each point represents an individual mouse from the two replicate studies (n = 10); horizontal lines denote median values; significance was calculated by the Kruskal-Wallis test with Dunn's correction for multiple comparisons. P-values of ≤0.05, ≤0.01, ≤0.001, and ≤0.0001 are represented as one, two, three, and four symbol characters, respectively.

significant increase in the production of IL-17A (Fig. 3G) and TNF-α (Fig. 3H) in any CD8$^+$ T-cells.

3.2. Heterologous vaccination induces polyfunctional T-cells

Polyfunctional T-cells are recognized as effector cells that provide higher protection against pathogens and enhanced memory cell functions. Higher levels of polyfunctional T-cell subsets have been directly related to improved vaccine efficacy in other models of intracellular pathogens, including *Borrelia* spp and *Salmonella* spp, as well as the disease activity in psoriatic arthritis (Infante-Duarte et al., 2000). For CD, polyfunctional T-cell responses provide more efficient control of *T. cruzi* infection and limit peripheral tissue damage (Albareda et al., 2013). Fig. 4 shows CD8$^+$ T-cells co-producing IFN-γ, IL-2, IL-17A, and TNF-α in response to Tc24-C4 protein stimulation.

Only heterologous vaccination with the Tc24 mRNA/protein vaccine

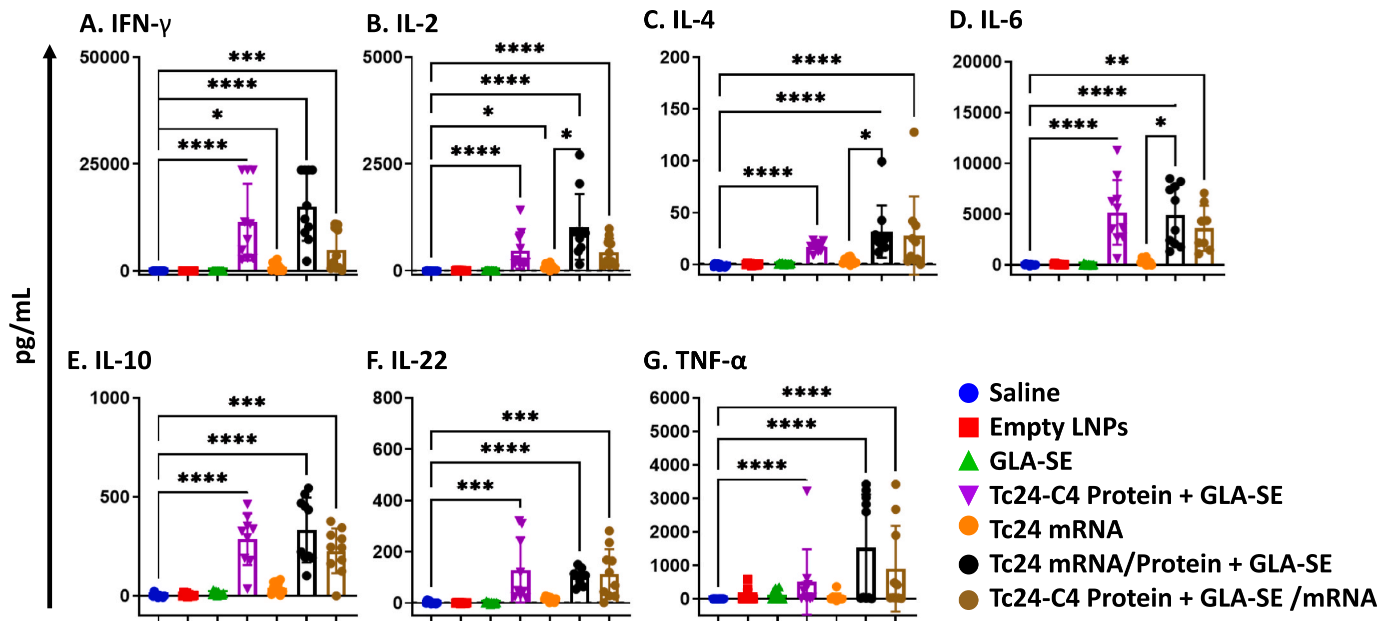


Fig. 5. Multiplex analysis of secreted cytokines by Luminex. Splenocytes were restimulated with 10 μg/μL Tc24-C4 protein for 48 h. The multiplex analysis measured secreted cytokines from these splenocytes. **A.** IFN-γ **B.** IL-2 **C.** IL-4 **D.** IL-6 **E.** IL-10 **F.** IL-22 **G.** TNF-α. The results are from two independent experiments. Each point represents an individual mouse from two replicate studies (n = 10); horizontal lines denote median values; significance was calculated by the Kruskal-Wallis test with Dunn's correction for multiple comparisons. P-values of ≤0.05, ≤0.01, ≤0.001, and ≤0.0001 are represented as one, two, three, and four symbol characters, respectively.

showed an increase in the co-production of TNF-α+IFN-γ (Fig. 4C) from CD8^{+} T-cells. Finally, polyfunctional CD8^{+} T-cells co-producing TNF-α+IL-17A (Fig. 4B), IFN-γ+IL-17A (Fig. 4D), IFN-γ+IL-2 (Fig. 4E) and IL-17A + IL-2 (Fig. 4F) were higher in both heterologous vaccination groups than in the homologous schemes.

3.3. *Heterologous vaccination induces a more favorable $T_H1/T_H2/T_H17$ cytokine profile compared to homologous vaccination*

Both heterologous vaccination strategies significantly increased IFN-γ (Fig. 5A), IL-2 (Fig. 5B), IL-4 (Fig. 5C), IL-6 (Fig. 5D), IL-10 (Fig. 5E), IL-22 (Fig. 5F), and TNF-α secretion (Fig. 5G), similarly to homologous vaccination with the Tc24-C4 protein vaccine. In contrast, homologous vaccination with the mRNA vaccine only significantly increased secretion of IFN-γ (Fig. 5A), IL-2 (Fig. 5B), and IL-4 (Fig. 5C). Overall, we conclude that heterologous vaccination induced a more robust Th1/Th2/Th17 profile than homologous vaccination with an mRNA vaccine. Furthermore, there were no significant differences between the levels of cytokines secreted when comparing the homologous Tc24-C4 protein vaccine groups to either heterologous vaccination group, indicating that a single dose of the Tc24-C4 protein vaccine is sufficient to obtain a similar profile of secreted cytokines.

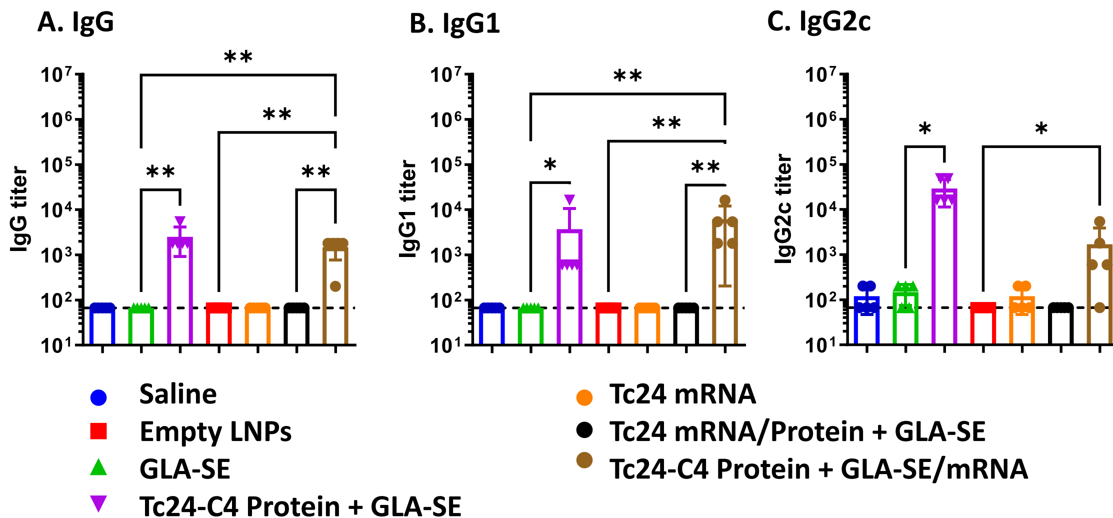


Fig. 6. Tc24-C4 specific antibody responses after prime vaccination. Serum antibody titers were assessed by ELISA 21 days after the prime vaccination. **A.** Tc24-C4-specific total IgG antibody titer. **B.** Tc24-C4-specific IgG1 antibody titer. **C.** Tc24-C4-specific IgG2c titer. Each point represents an individual mouse (n = 5). Horizontal lines denote median values. Dotted lines indicate the baseline. Significance was calculated by the Kruskal-Wallis test with Dunn's correction for multiple comparisons. P-values of ≤0.05, ≤0.01, ≤0.001, and ≤0.0001 are represented as one, two, three, and four symbol characters, respectively.

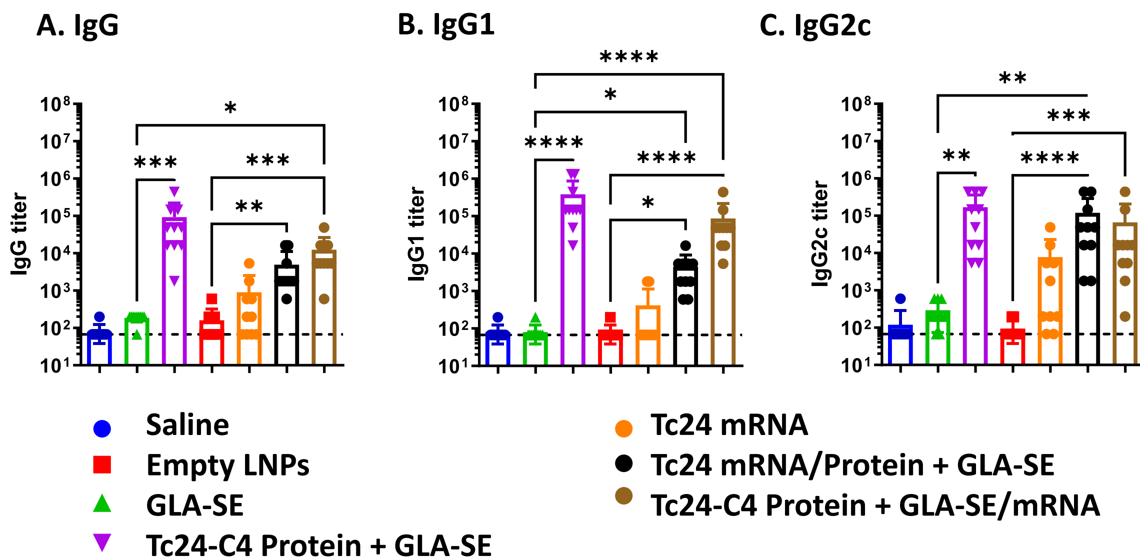


Fig. 7. Tc24-C4 specific antibody responses after boost vaccination. Antigen-specific antibody titers were assessed by ELISA 19 days after the boost vaccination or 40 days after the prime vaccination **A.** Tc24-C4-specific Total IgG antibody titer. **B.** Tc24-C4-specific IgG1 antibody titer. **C.** Tc24-C4-specific IgG2c. The results are from two independent experiments. Each point represents an individual mouse (n = 10). Horizontal lines denote median values. Dotted lines indicate the baseline. Significance was calculated by the Kruskal-Wallis test with Dunn's correction for multiple comparisons. P-values of ≤0.05, ≤0.01, ≤0.001, and ≤0.0001 are represented as one, two, three, and four symbol characters, respectively.

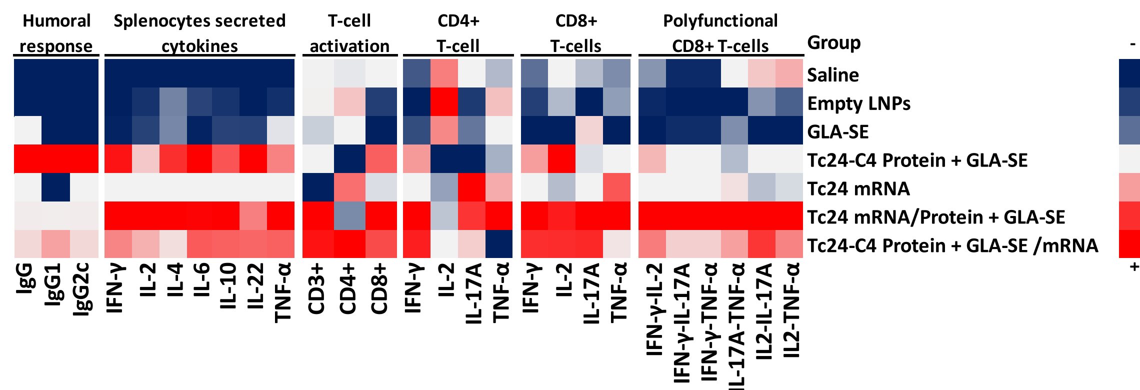


Fig. 8. Heat map visualization applied to the immunological evaluation of Tc24-vaccinated mice. Six parameters are shown as (a) Humoral response (b), Splenocyte secreted cytokines (c), T-cell activation (d), $CD4^+$ T-cell (e), $CD8^+$ T-cell, and (f) Polyfunctional $CD8^+$ T-cells. Each column corresponds to immunoglobulins, T-cells population, or cytokine(s), while rows correspond to the median of each group evaluated. The colors indicate secretion titers (blue = low, red = high). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Protein vaccines are needed to have antigen-specific antibody responses

The total IgG antibody level was measured to evaluate the overall humoral response, and IgG1 and IgG2c isotype levels were measured to assess a balanced humoral response three weeks after prime vaccination. Our results showed a Tc24-C4 specific IgG antibody response in all groups primed with the Tc24-C4 protein vaccine (Fig. 6A). The IgG titers were significantly higher with the Tc24-C4 protein vaccine (2520) and with the heterologous Tc24-C4 protein/mRNA vaccine (1480) than in the groups primed with the Tc24 mRNA (Fig. 6 A). A similar trend was found for IgG1, with the Tc24-C4 protein vaccine producing a titer of 3720 and the heterologous Tc24-C4 protein/mRNA vaccine with a titer of 6120 (Fig. 6 B). Finally, the same pattern was obtained in the evaluation of the IgG2c titers where the only two groups with significantly higher titers were the Tc24-C4 protein vaccine (29160) and the heterologous Tc24-C4 protein/mRNA vaccine (1693) (Fig. 6C). At this point, both groups had been treated equivalently with only the Tc24-C4 protein vaccine.

A second evaluation of the humoral immune response was conducted 19 days after the boost vaccination or 40 days after the prime vaccination, with either the homologous or heterologous vaccine. The total IgG and IgG1 titers were significantly higher in the groups that received at least one dose of the Tc24-C4 protein vaccine (Fig. 7A). Homologous vaccination with the Tc24-C4 protein vaccine induced the highest IgG titers (92520), followed by heterologous protein/mRNA vaccination (12480), mRNA/protein vaccine (4920), and the lowest levels of IgG were found in the homologous Tc24 mRNA vaccination group (907) compared with the saline group (80) (Fig. 7 A). Similarly, IgG1 titers were highest in the homologous Tc24-C4 protein vaccine group (385560), followed by the heterologous vaccinations, Tc24-C4 protein/mRNA vaccine (86400), Tc24 mRNA/protein vaccine (4440), but only two mice developed measurable titers in the homologous Tc24 mRNA vaccine group (414) compared with the saline group (80) (Fig. 7B). Finally, significant IgG2c titers were consistent with the results of the IgG and IgG1, were highest in the homologous Tc24-C4 protein vaccine group (169560), followed by the heterologous vaccinations, Tc24 mRNA/protein vaccine (120240), Tc24-C4 protein/mRNA vaccine

(66080), but only two mice developed measurable titers in the homologous Tc24 mRNA vaccine group (7813) compared with the saline group (80) by 40 days post-prime (Fig. 7 C).

Finally, to show the magnitude of the effect of the heterologous vaccine, we generated a heat map to visualize and summarize the data obtained using the median value of each parameter (Fig. 8). We found that the heterologous vaccine using the Tc24 mRNA to prime and the protein vaccine to boost induced higher levels of polyfunctional $CD8^+$ T-cells, $CD4^+$ IFN-γ, $CD8^+$ IFN-γ, $CD8^+$ IL-2 and $CD8^+$ TNF-α cytokine-producing T-cells, secreted Th1/Th2/Th17 cytokines from splenocytes, and T-cell activation ($CD3^+$ $CD25^+$). In contrast, the homologous Tc24-C4 protein vaccine induced the highest humoral response and secretion of cytokines from splenocytes. However, the homologous protein vaccine induced weaker cellular responses when compared with the heterologous vaccines, where the Tc24 mRNA vaccine plays an important role. The lack of humoral response in the homologous Tc24-mRNA shows that the presentation mechanisms are mainly via MHC-I, indicating the need to improve the construct's design to obtain the secreted protein that facilitates the presentation via MHC-II. Interestingly, the homologous Tc24-mRNA vaccination scheme does not have a higher cellular response than the heterologous scheme, indicating that the heterologous presentation of Tc24 antigen as both protein and mRNA induces a more robust profile.

4. Discussion

This is the first study demonstrating the immunogenicity of *T. cruzi* antigens delivered as mRNA vaccines, either as a homologous vaccine or in a heterologous prime/boost approach. Our data demonstrate a similar immunogenicity profile of these Tc24-specific vaccines in a C57BL/6 mouse model as seen in earlier studies with protein-alone in Balb/C mice (Barry et al., 2016, 2019). We showed improved immunogenicity using a heterologous mRNA/protein vaccine and demonstrated that heterologous vaccination induced significantly increased levels of activated $CD4^+$ and $CD8^+$ T-cells, increased cytokine-producing $CD4^+$ and $CD8^+$ T-cells, and a balanced Th1/Th2/Th17 secreted-cytokine profile. Importantly, we observed that heterologous vaccination-induced significantly increased polyfunctional $CD8^+$ T-cells that simultaneously produced various cytokines.

We determined that the heterologous mRNA/protein vaccination strategy induced significantly increased $CD8^+$ cells that co-produced multiple cytokines, including IFNγ, TNFα, IL-2, and IL-17A. Polyfunctional T-cell immune responses have been associated with more effective control of *T. cruzi* infection (Sanchez Alberti et al., 2020). Therefore, polyfunctional T-cell priming would be a desirable attribute for a T-cell-based vaccine against *T. cruzi*, considering that it elicits a higher effector function and a greater long-term memory potential. In agreement with this observation, our heterologous vaccination strategy (mRNA/protein) induced polyfunctional $CD8^+$ T-cells secreting both IFN-γ and TNF-α, which have been shown to have improved cytolytic activity compared with cytotoxic $CD8^+$ T-cells that secrete IFN-γ alone (Lichterfeld et al., 2004). All these might contribute to a more potent immune response and better control of infection. In addition, our results showed higher levels of another population of polyfunctional T-cells co-producing IL-17A and IL-2 or IFN-γ induced by heterologous vaccination. In the case of IL-17A, during *T. cruzi* infection, the secretion of this cytokine triggers NADPH oxidase, which in turn produces reactive oxygen species (ROS), protecting cells by killing intracellular parasites (Cai et al., 2016). Th1-biased polyfunctional T-cells (IFN-γ and TNF-α-producing) and Th17-biased polyfunctional cells (IL-17A-producing) can maintain pathogen control while minimizing pathology (Khakhum et al., 2021). Early production of IL-17 promotes a Th1 response. However, in the case of chronic tuberculosis, IFN-γ is necessary to regulate the damage through Th17 responses (Khader et al., 2007; Cruz et al., 2010). These findings suggest that our heterologous vaccines induce Th1/Th17 responses that could better control *T. cruzi* infection in affected tissues.

Our results showed that the vaccine increases the percentage of $CD8^+CD25^+$ cells. Effector and memory $CD8^+$ T-cells are critical for *T. cruzi* control. Enhanced expression of CD25 facilitates the response of $CD8^+$ T-cells to IL-2 and promotes $CD8^+$ T-cell survival and their ability to increase in number upon secondary antigen encounter (Cox et al., 2013). IL-2 can enhance natural killer (NK)-cell activity that itself could contribute to the early control of infection following challenge and could promote the expansion of T-cells in an autocrine or paracrine manner, enhancing $CD8^+$ T-cells memory function (Sandberg et al., 2001; Seder et al., 2008). All these together suggest that heterologous vaccination will enhance key effector and memory $CD8^+$ T-cells. On the other hand, the role of regulatory T-cells ($CD4^+CD25^+Foxp3^+$) during *T. cruzi* infection is not completely understood (Fresno and Gironès, 2018). Studies in chronic and acute mouse models suggested these cells may play a role in limiting tissue inflammation during infection, and a depletion of these cells can exacerbate acute lethal infections (Kotner and Tarleton, 2007; Mariano et al., 2008). Other reports using $CD4^+CD25^+Foxp3^+$ cells induced by vaccination with the amastigote-derived rSSP4 antigen showed that these cells controlled early cardiac inflammation and prolonged survival in BALB/c mice infected with the H8 Yucatan strain of *T. cruzi*, but concomitantly exacerbated parasite burdens (Flores-García et al., 2013). However, while we did not show an increase in the number of $CD4^+CD25^+$ cells, the $Foxp3^+$ marker was not assessed.

The immune response profile triggered by the presentation of the Tc24-C4 protein displayed a Th1/Th2/Th17 bias with a balanced cytokine profile. The robustness of the immune response was obtained with one and two doses of the Tc24-C4 protein vaccine. Previously, we demonstrated in an acute infection model that therapeutic vaccination with the Tc24-C4 protein vaccine induced a balanced cytokine profile accompanied by reduced tissue parasite burdens as well as reduced tissue inflammation and fibrosis (Cruz-Chan et al., 2021). Patients with chagasic cardiopathy present a proinflammatory profile with elevated TNF-α, IFN-γ, and IL-12; in comparison with the asymptomatic patients where the profile is mainly anti-inflammatory with increased IL-10 and IL-17A, the balance of the immune response plays an important role in determining the clinical presentation and the severity of the outcome (Poveda et al., 2014). This suggests that the heterologous vaccination strategy will likely reduce pathology.

Interestingly, the presentation of the Tc24 antigens as an mRNA improves the cellular response on the heterologous but not in the homologous scheme compared to the presentation as a homologous protein vaccine. Vaccines for CD require T-cells to mediate protection and increase antigen-specific production of effector molecules, including IFN-γ and TNF-α, which enhance killing activities and the balance of the cytolytic mechanisms or secretion of cytokines by regulatory T-cells producing inhibitory cytokines such as IL-10 (Seder et al., 2008). While such effects might be achieved through a homologous mRNA immunization series, mixing it up with protein provides added benefits. For instance, our results show that mice that received the homologous Tc24-C4 protein vaccine had the highest antigen-specific IgG, IgG1 and IgG2c antibody titers at day 40, followed by those who received the heterologous protein/mRNA vaccine, those that received the heterologous mRNA/protein vaccine, and those that received the homologous mRNA vaccine. A balanced humoral response between IgG1/IgG2c isotypes is essential to control the infection; an increase in the IgG1 isotype could have some positive effect on clearing the parasite through opsonization and phagocytosis (el Bouhdidi et al., 1994). These results suggest that an additional mechanism of protection induced by vaccines containing protein antigens is through the production of specific opsonizing antibodies. Therefore, high levels of IgG1 associated with the Tc24-C4 protein + GLA-SE antigen could be crucial for protection from infection.

Our results showed a robust humoral response which indicates that activated $CD4^+$ cells interacted with B cells to induce antibodies.

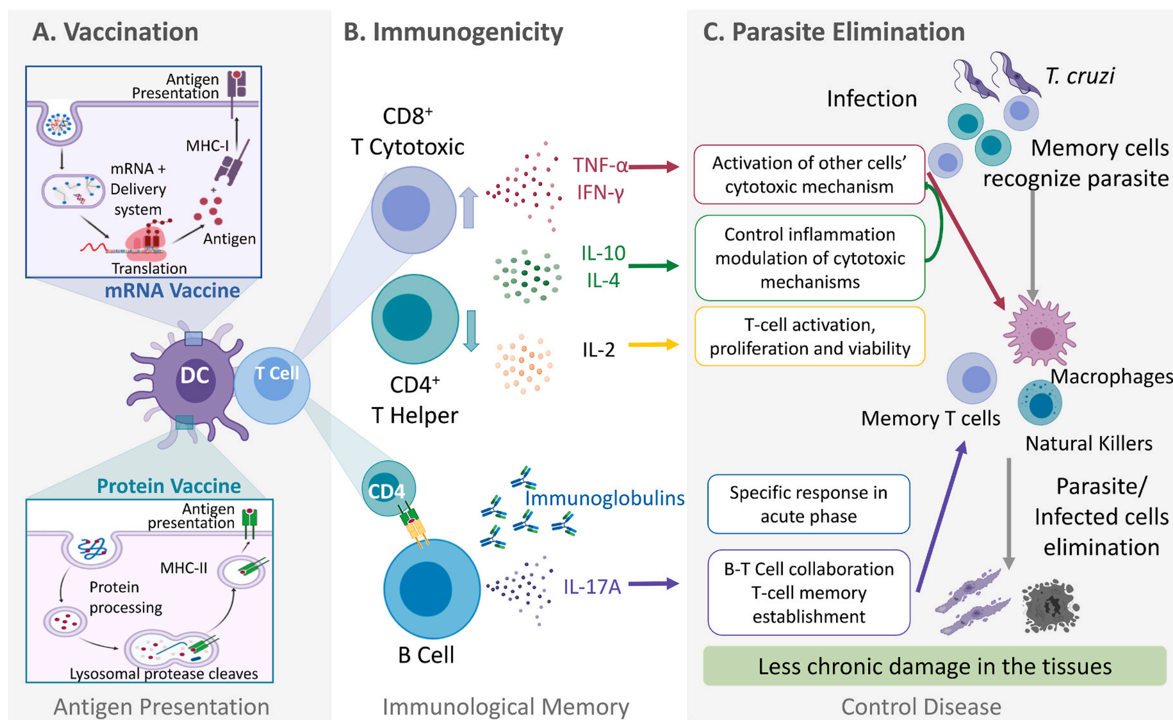


Fig. 9. Proposed mechanism of the response to heterologous vaccination. A. Vaccination. mRNA and protein vaccines are taken up by dendritic cells. The mRNA is translated to a protein antigen, then broken down into peptides that are subsequently presented to naïve T-cells on MHC Class I molecules. The lysosomal protease processes the protein vaccine and the antigen is cleaved to be presented to naïve T-cells on MHC Class II molecules. **B. Immunogenicity**. Antigen presentation triggers T-cell activation (CD25$^+$), proliferation, and differentiation into different subsets, CD4$^+$ helper T-cells, CD8$^+$ cytotoxic T-cells, and a group of memory T-cells. Memory T-cells will persist in the circulation and the tissues for a long time and confer immunological memory. **C. Parasite Elimination**. When these vaccinated animals are challenged with the parasite (*T. cruzi*), memory cells recognize the parasite. That recognition activates the CD4$^+$ and CD8$^+$ T lymphocytes. CD4$^+$ enhances or modulates the immune response mechanisms against *T. cruzi* infection. CD8$^+$ T lymphocytes play a fundamental role in controlling parasitemia and eliminating cells infected with *T. cruzi*. The control of the disease requires a balanced response (Cytokines) in which there is a compromise between the activation of microbicidal functions (Proinflammatory cytokines) and the control of inflammatory damage (Modulatory Cytokines).

However, Tc24 as a protein or mRNA has not impact on CD25 expression in the CD4$^+$ compartment. To understand the kinetics of CD4$^+$ T cells, we will need to add early activation markers, CD69, or evaluate CD25 at a later time point. Another hypothesis is that similar to some microbial antigens our Tc24 antigen can activate B cells directly without requiring the help of CD4 T-cells, which can lead to the production of IgG (Janeway et al., 2714a). Further studies are needed to understand the complex interactions between B cells, CD4 T helper cells, and other immune system components to identify strategies for inducing strong and effective immune responses.

Our study demonstrated that a solid immune profile is triggered by the Tc24 antigen as homologous vaccine presentation as a protein or mRNA and has a more robust immune profile when the antigen is presented as a heterologous Tc24-C4 protein/mRNA vaccine shown in the summary heat map from our result (Fig. 8). These data suggest that the heterologous vaccination approach takes advantage of the different antigen processing and presentation methods for protein and mRNA antigens by APCs to induce a more robust immune response, as illustrated in our proposed model in Fig. 9. When protein vaccines are taken up by APCs, the protein antigen is processed by the lysosomal and cleavage to be presented to the CD4$^+$ T cell on MHC Class II molecules (Robinson and Delvig, 2002); the presentation of APC T-cell is followed by the activation of B-cells by antigen and specialized T helper cell which creates antibodies and defensive white blood cells (Janeway et al., 2714b). In contrast, when mRNA vaccines are taken up by APCs the mRNA is translated to protein inside the cytosol of the APC and then immediately digested into peptides that are presented via MHC-I to the T-cells giving a cellular response but a low humoral response (Versteeg et al., 2019)(Fig. 9). We were interested in determining if the order of presenting the antigen, either on MHC Class II or MHC Class I molecules, would stimulate a different immune profile. Our data shows that after prime vaccination presenting protein first stimulates higher IgG1 titers compared to presenting mRNA first, while after boost vaccination, the order of antigen presentation did not significantly change the humoral or T cell immune response. Furthermore, heterologous prime mRNA and boost protein vaccination improved the immune response, especially through the elicitation of strong T-cell responses.

With this study, we demonstrated a broad and robust immune response using a heterologous vaccination scheme with the Tc24 antigen as a recombinant protein and as mRNA. Our study highlighted that the heterologous vaccine elicited both humoral and cellular immune responses, required for complete protection against *T. cruzi*. Some limitations of this study include that further evaluation of cytolytic molecules (perforin, Granzyme B) to confirm the functionality of the proposed cytotoxic CD8$^+$ T-cells is needed. Since we only evaluated early time points after the boost vaccination, we do not know with any certainty if this strategy induced memory cells. Studies to evaluate optimized mRNA constructs are ongoing, including evaluating constructs containing a signal peptide that allows secretion of protein antigen *in vivo* and constructs with improved *in vivo* transfection and

translation to increase the immune response's magnitude further. Future studies evaluating the efficacy of heterologous vaccination as a prophylactic or therapeutic vaccine in a mouse model are necessary to understand if the immune response profile obtained here is also superior in controlling the disease.

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CRediT authorship contribution statement

CP, KJ, JP, PJH, US, and MEB conceived and designed the study. CP, AL, RA, DMN, MJV, and LV conducted the experiments. RK and YLC for plasmid linearization and mRNA production. CM and F.T LNPs formulation. CP, AL, and RA collected data. CP, KMJ, and JP analyzed the data. JP and MEB provided reagents. CP, AL, KMJ, JP, US, PJH, and MEB wrote the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Institutional reports financial support was provided by Robert J Kleberg Jr and Helen C Kleberg Foundation. All Authors are part of a team of scientists advancing research towards the development of a therapeutic Chagas disease vaccine. MEB and PJH are listed among the inventors on a Chagas disease vaccine patent, submitted by Baylor College of Medicine. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the review article apart from those disclosed.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crimmu.2023.100066.

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